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UNDERSTANDING THE BIOKINETICS OF IBUPROFEN AFTER SINGLE AND REPEATED TREATMENTS IN RAT AND HUMAN *IN VITRO* LIVER CELL SYSTEMS

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Abstract

Common *in vitro* toxicity testing often neglects the fate and intracellular concentration of tested compounds, potentially limiting the predictability of *in vitro* results for *in vivo* extrapolation. We used *in vitro* long-term cultures of primary rat (PRH) and human hepatocytes (PHH) and HepaRG cells to characterise and model the biokinetic profile of ibuprofen (IBU) after single and daily repeated exposure (14 days) to two concentrations. A cross-model comparison was carried out at 100 µM, roughly corresponding to the human therapeutic plasma concentration. Our results showed that IBU uptake was rapid and a dynamic equilibrium was reached within 1 or 2 days. All three cell systems efficiently metabolised IBU. In terms of species-differences, our data mirrored known *in vivo* results. Although no bioaccumulation was observed, IBU intracellular concentration was higher in PRH due to a 10-fold lower metabolic clearance compared to the human-derived cells. In HepaRG cells, IBU metabolism increased over time, but was not related to the treatment. In PHH, a low CYP2C9 activity, the major IBU-metabolising CYP, led to an increased cytotoxicity. A high inter-individual variability was seen in PHH, whereas HepaRG cells and PRH were more reproducible models. Although the concentrations of IBU in PRH over time differed from the concentrations found in human cells under similar exposure conditions.

Keywords: Biokinetics, Ibuprofen, *in vitro*, Primary rat hepatocytes, Primary human hepatocytes, HepaRG cells

Abbreviations

ADR - adverse drug reaction

bcell - biliary cell

BSA - bovine serum albumin

C_{cell} - concentration in cell lysate

C_{max} - human therapeutic peak plasma concentration

C_{med} - concentration in assay medium

CV- coefficient of variation

CYP - cytochrome P450

DME - drug metabolising enzyme

DMEM - Dulbecco modified eagle medium

DMSO - dimethyl sulphoxide

FBS - foetal bovine serum

F_{in} - entry rate flow for one cell

F_{out} - exit rate flow for one cell

HMM - hepatocyte maintenance medium

IBU - ibuprofen

ITS - insulin transferrin selenium

k_1 - rate constant for binding to medium proteins

k_2 - rate constant for unbinding from medium proteins

K_m - Michaelis-Menten constant

LOD- Limit of detection

LOQ – Limit of quantification

MCMC - Markov-chain Monte Carlo

N_{cell} - number of cells in the assay system

NOAEC – No observed adverse effect concentration

OECD - Organisation for Economic Co-operation and Development

PHH - primary human hepatocytes

PBPK - physiologically-based pharmacokinetics

PRH - primary rat hepatocytes

Q_{cell} - total quantity in cell lysate

Q_{med} - total quantity in assay medium

Q_{prot} - total quantity bound on protein

SD - standard deviation

SW - sandwich

$t_{1/2}$ - half-life

TC - toxic concentration

T_{max} - time to reach C_{max}

V_{cell} - volume of cells

V_{max} - maximal metabolic rate

V_{med} - volume of assay medium

1 Introduction

Attrition during drug discovery and development is a major hurdle to the launch of a drug and lack of efficacy and unacceptable toxicity are the two major reasons (Kola and Landis, 2004). Usually, the potential risk for human health of pharmaceuticals is assessed in the early phases of development on the basis of animal testing. However, the extrapolation of observed adverse drug reactions (ADRs) from animals to humans is often difficult. The discrepancies can be frequently attributed to different kinetic behaviours of the compound in the different species (Shanks et al., 2009).

In the last decades, *in vitro* models have improved substantially, resulting in applications accepted as valuable tools to characterise and optimise compounds in terms of efficacy and safety. Most of these *in vitro* models apply a single exposure to deliver discrete information on single endpoints. By contrast, more complex *in vitro* models, for the identification of systemic effects, lack acceptance mainly because they poorly correlate with *in vivo* data (Adler et al., 2011). Differences between *in vitro* and *in vivo* kinetics have been considered as one of the main reasons. Despite this, the implementation of biokinetic information in *in vitro* systems has been mainly ignored. As a consequence, an observed effect indicated by e.g. EC_{50} (Effective Concentration causing the 50% of the effect) or NOAEC (No Observed Adverse Effect Concentration) *in vitro* is typically assigned to the applied nominal concentration of the test item, assuming that 100% is available within the cell. On the contrary, a number of abiotic processes affects the fraction of a test chemical that is available for uptake into cells or tissue, reducing its bioavailability. These processes include compound solubility, volatility, stability in aqueous solutions, binding to membrane lipids and proteins in cell culture medium or adsorption to plastic devices. Furthermore, biotic processes, such as mechanisms of cell uptake/extrusion, metabolism, intracellular bioaccumulation (of parent and/or metabolites) as well as saturation of these processes can influence the compound's biokinetic behaviour, affecting the biologically effective dose of test chemical, able to interact with the target or cause toxicity. This hampers the interpretation of *in vitro* data to predict *in vivo* dose–response relationships and compare the true toxic potency of test compounds (Groothuis et al, 2013). Thus, the intracellular concentration is a much more relevant

parameter to enable the derivation of a NOAEC *in vitro*. This NOAEC can be then transformed to *in vivo* doses using appropriate modelling techniques, such as physiologically-based pharmacokinetics (PBPK) modelling.

Previous groups have shown that adsorption to plastic devices (Tirelli et al., 2007), binding to macromolecules in the medium (Guelden et al., 2001; Seibert et al., 2002), evaporation of the chemical (Kramer et al. 2012) and the number of cells in the cell system (Guelden et al., 2001; Guelden et al., 2010) influence the actual biologically effective concentration and thus the cytotoxic potential of a compound. A recent paper reviewed a number of factors affecting bioavailability of test chemicals in *in vitro* assays and different dose metrics for *in vitro* setups (Groothuis et al, 2013).

To further support this concept, the application of a recently developed model to a set of hypothetical chemicals as well as to 1194 real substances (predominantly from the ToxCast chemical database) shows that the potential range of concentrations and chemical activities under assumed test conditions can vary by orders of magnitude for the same nominal concentration (Armitage et al. 2014).

There is an urgent need for predictive *in vitro* models to identify ADRs in the early phases of drug development, especially for the liver. As the main drug metabolising organ, the liver plays a central role in drug-induced toxicities. Furthermore, repeated drug administration is a more relevant exposure scenario for therapeutics, being usually evaluated in specified *in vivo* repeated-dose toxicity testing. In order to mimic repeated exposures *in vitro*, models retaining *in vivo* characteristics for a sufficiently long time frame should be used. Both hepatotoxicity and repeated exposure were addressed in this work by using different long-term hepatic culture systems.

Primary hepatocytes are the gold standard for metabolism studies because these cells retain *in vivo*-like activities of drug metabolising enzymes (DMEs) (Guillouzo, 1998; Hewitt et al., 2007; Tuschl et al., 2008). However, monolayer cultures of primary hepatocytes lose the activity of some liver-specific enzymes within a few days (Guillouzo, 1998; Tuschl et al., 2009). By contrast, primary rat and human hepatocytes (PRH and PHH, respectively) cultured in a sandwich (SW) configuration with defined medium, maintain their metabolic capacities at acceptable levels over a prolonged time period

(Parmentier et al., 2013; Tuschl et al., 2009). The cholangio-hepatocarcinoma derived cell line HepaRG™ has proven itself valuable for many applications, including the prediction of metabolism-dependent hepatotoxicity (Aninat et al., 2006; Anthérieu et al., 2012). This human-derived cell line is a promising system, because after proliferation and differentiation phases it holds adequate and rather stable activity of DMEs throughout long-term culture.

The EU FP7 Project Predict-IV aimed to provide an improved predictability of the non-clinical safety testing by using *in vitro* tests, proposed to integrate dynamics and biokinetics in *in vitro* models after repeated exposure. This paper describes some of the obtained results comparing the three hepatic models described above to study the kinetic behaviour of ibuprofen (IBU), after acute and long-term repeated treatment.

IBU, a non-steroidal anti-inflammatory drug, seldom inducing ADRs in the liver, has been used as model compound, selected on the basis of its physicochemical and metabolic properties.

To the best of our knowledge the *in vitro* biokinetics after single (d0/1) and repeated exposures (d13/14) of a drug are here described for the first time. The integration of biokinetics to well-established rat and human long-term liver culture systems addresses most of the current issues of *in vitro* systems described above. The approach further includes the application of PK modelling, by integrating the kinetic experimental parameters obtained in the different *in vitro* systems, and being a fundamental tool for the extrapolation of *in vitro* data to the *in vivo* situation.

2 Materials and Methods

2.1 Chemicals and Reagents

IBU was purchased from Sigma-Aldrich (Steinheim, Germany; St. Quentin-Fallavier, France) or Calbiochem (Darmstadt, Germany). For the culture of the PRH in sandwich configuration Collagen from rat tail tendon (Roche, Mannheim, Germany) was used, while PHH were covered with Geltrex™ from Gibco® (Thermo Fisher Scientific, Illkirch, France). The perfusion buffer components were from Merck Chemicals (Merck KGaA) and AppliChem (both Darmstadt, Germany) or Sigma-Aldrich (Steinheim, Germany; St. Quentin-Fallavier, France). DMEM/F12 GlutaMAX™, Williams' E medium, Sodium-pyruvate, ITS-G (100x), Gentamycin, Glutamine, Trypsin/EDTA and PBS were obtained from Gibco® (LifeTechnologies, Darmstadt, Germany; Thermo Fisher Scientific, Illkirch, France). The culture medium, HMM, used with PHH was purchased from Lonza (Verviers, Belgium). Foetal bovine serum (FBS) was from HyClone®, Gibco® or Perbio (Thermo Fisher Scientific, Waltham (MA), USA or Illkirch, France); hydrocortisone hemisuccinate was from Upjohn Pharmacia (Guyancourt, France). Further reagents were from Sigma-Aldrich (Steinheim, Germany; St. Quentin-Fallavier, France) included penicillin/streptomycin, insulin, BSA, dexamethasone, percoll® and DMSO.

For IBU quantification analytical grade chemicals were obtained from commercially available sources. The Milli-Q water purification system (Millipore, Merck KGaA, Darmstadt, Germany) was used to obtain deionised water.

2.2 Cell culture

2.2.1 Primary rat hepatocytes

Care and use of laboratory animals was in agreement with the German guidelines and approved by the ethics committee. Isolation of rat hepatocytes from male Wistar rats (Harlan Laboratories, Rossdorf, Germany) followed a modified two-step perfusion technique described by Seglen (Seglen, 1976). Overall, only hepatocyte preparations with more than 85% viability (determined via trypan exclusion method) were used. 30,000 viable cells were seeded onto collagen I coated 96-well plates in serum-containing culture media (DMEM/F12 GlutaMAX™, 100 units/mL penicillin, 100 µg/mL

streptomycin, 1 mM sodium pyruvate, 10% FBS and 5 µg/mL insulin). Following an attachment phase of 4 h the culture medium was replaced with serum-free culture medium media (DMEM/F12 GlutaMAX™, 100 units/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate, 100 nM dexamethasone, 0.44 mg/L BSA, ITS-G) and IBU treatment was started 24h after seeding. For the SW culture format, the cells were seeded in serum-containing culture media on a gelatinised layer of collagen (prepared at 37°C ≥ 1 h) at a density of 0.25 and 1.5 x 10^6 viable cells per well in a 24- and 6-well plate (BD Falcon™, Heidelberg, Germany), respectively. After four hours the culture medium was replaced with fresh serum-containing media. On the subsequent day the top collagen stratum was applied and after its full gelatinisation (at 37°C ≥ 1.5 h) serum-free culture medium media was added. IBU treatment started on day 3 after seeding.

2.2.2 Primary Human Hepatocytes

All experiments were performed with permission of the National Ethics Committee (France) and regulatory authorities. Liver biopsies (20-100 g) were received from surgical operations of patients with different pathologies (Table 1), whereas the obtained tissue was removed with a safety margin from the aberrant section. LeCluyse and Alexandre (2010) delineate the two-step perfusion procedure which was applied to isolate the PHH from the liver resections.

Viability of the PHH suspension, obtained directly after isolation, was assessed via trypan blue exclusion method and only preparations holding viability greater than 70% were used for cell culture experiments. The cells were seeded at a density of 2 x 10^6 viable cells per well of a collagen-coated 6-well BD Biocoat® plate (Dutscher, France) for kinetic studies or at a density of 0.3 x 10^6 viable cells per well of a 24-well plate (Biocoat®, Dutscher, France) for CYP450 activity measurements. The seeding medium was Williams' E medium containing 50 µg/mL gentamycin, 10% FBS, 1 µM dexamethasone and 4 µg/mL insulin. Following an overnight incubation the monolayer culture was covered with a preparation of 350 µg/mL Geltrex™ in serum-free culture media (HMM, 50 µg/mL gentamycin, 100 nM dexamethasone and 1x ITS-G). The Geltrex™ overlay was renewed every 3-4 days. IBU treatment was started two days after seeding.

2.2.3 HepaRG cells

The liver tumour derived HepaRG cells were cultured and differentiated as previously described by Gripon et al. (2002). Briefly, the HepaRG cells (at passage 12) were cultured in growth medium (Williams' E supplemented with 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, 5 µg/mL insulin, 2 mM glutamine, and 50 µM hydrocortisone hemisuccinate), which was refreshed three times a week. After two weeks the growth medium was supplemented with 2% DMSO for additional two weeks, resulting in differentiated HepaRG cell cultures. At that time the cultures contained hepatocyte-like and primitive biliary cells at around the same percentage (Cerec et al., 2007; Aninat et al., 2006) and were ready for treatment with IBU. Two days before treatment start the cells ($2x10^6$ per well) were shifted to a medium containing 2% FBS and 1% DMSO. The percentage of FBS was lowered to 2% to minimise protein binding of the test item, without altering the cell viability and functionality (data not shown).

2.3 Determination of CYP450s activity in PHH

Basal CYP450s activities were determined in PHH cultures from the three donors used for kinetic experiments. After attachment, cell monolayers were washed with warm phosphate buffered saline (PBS) during 15 min at 37°C, and thereafter incubated at 37°C under air/CO2 (95/5 %) in DMEM with Glutamax I supplemented with insulin (4 mg/mL), dexamethasone (1 µM) and antibiotics, containing the substrates as a cocktail: 3 µM midazolam, 100 µM bupropion and 10 µM diclofenac for CYP3A4/5, CYP2B6 and CYP2C9 respectively, as described by Kanebratt et al. (2008) or with ethoxyresorufin for CYP1A1/2. At the end of the incubation period, supernatants were collected and stored at -80°C until analysis. The metabolites midazolam 1'-hydroxylation (CYP3A4/5), diclofenac 4-hydroxylation (CYP2C9) and bupropion-hydroxylation (CYP2B6) were determined by LC-MS/MS and resorufin by fluorescence, as described by Alexandre et al. (2012).

2.4 Abiotic processes

2.4.1 Stability and solubility of the test compound

The stability of IBU in both aqueous solutions and in DMSO (the vehicle used to prepare stock solutions), was checked in preliminary assays, mimicking the actual experimental conditions. Distilled water, culture media and IBU solutions in DMSO were kept at 37°C for different incubation times in glass lab-wear and plastic tubes and analysed by HPLC.

2.4.2 Cross-contamination among wells

In the adjacent wells to those containing IBU at the applied concentrations, a corresponding amount of DMSO (vehicle control) was added. At the end of the experiment, the content of these control wells was stored at -80°C until HPLC analysis. The IBU content in these control wells served as an indication of possible contamination across wells due to IBU evaporation.

2.4.3 Adsorption to plastic devices

In order to measure adsorption to the plastic, once the content of each well was removed, the wells were washed twice with PBS before adding 2 mL methanol. The plate was sealed with Parafilm, and kept on a horizontal shaker (350 rpm) at room temperature for 2 h before transferring the complete volume into a LoBind tube.

2.5 Sequestration by the matrix

To determine the amount of IBU possibly sequestered by proteins in the collagen or Geltrex™ matrix used for PRH and PHH SW cultures, repeated exposure experiments were conducted with the same schedule of the main experiment but in the absence of cells ('blank experiment'). Here, corresponding volumes of collagen I (PRH) and Geltrex™ (PHH) were exposed to high and low IBU concentrations or DMSO vehicle control. Sampling was performed on the first (0/1) and last (13/14) day of treatment, at two time points (2 min and 24 h).

2.6 Assessment of cell viability after IBU exposure

Preliminary experiments were performed to determine the cytotoxicity of IBU. For all applied assays, the results were expressed as a percentage versus the vehicle treated control.

In PRH, the dose finding was performed in three stages (Table 2), starting with treatment with a broad concentration range in 96-well plate ML culture for 24 h. After the pre-screen, three biological

replicates in 24-well plate SW culture were treated for 1, 3 and 14 days to assess a preliminary TC_{10} (the concentration causing 10% cytotoxicity, in the specific case measured as ATP depletion). Finally, three concentrations around the determined TC_{10} were tested in 6-well plate SW cultures, the format for the final experiments, for 14 days repeated exposure.

The cell viability was determined via ATP levels that were measured using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega®) according to the manufacturer's protocol including slight modifications. Briefly, after the corresponding exposure time cell cultures in 96-, 24- or 6-well plates were incubated with 100 µL, 600 µL or 900 µL CellTiter-Glo® Reagent for 2 min on an orbital shaker. After 10 min the luminescence signal was measured.

In PHH, preliminary assessment of IBU cytotoxicity was performed in one culture (donor S0302V, not further used for kinetic testing): 0.05 x 10^6 viable cells were seeded onto collagen I coated 96-well Biocoat® plates (Dutscher, France) in Williams' E medium supplemented with 10% FBS, 4 µg/mL insulin, 1 µM dexamethasone and 50 µg/mL gentamycin. Following an overnight incubation, the cell monolayer was covered with 350 µg/mL Geltrex™ in culture medium without FBS for 24 h. Six IBU concentrations were tested, corresponding to the concentrations applied to PRH in 24-well SW cultures (see Table 2). A MTT test was performed after 1, 3 and 14 days of treatment. Here, cells were incubated with 1 mg/mL thiazolyl blue tetrazolium bromide for 30 min, the supernatant was replaced by 100 µL DMSO per well. After gentle shaking, the absorbance was measured at 595 nm.

For IBU cytotoxicity determinations, 2.5 x 10^5 HepaRG cells were seeded per well in 96-well plates as described previously (Aninat et al., 2006). Briefly, after 1, 3 and 14 days of treatment, medium was removed and serum-free medium containing MTT (0.5 mg/mL) was added to each well and incubated for 2 h at 37°C. After removal of the incubation solution, water-insoluble formazan was dissolved in DMSO and absorbance was measured at 540 nm.

2.7 IBU exposure for kinetic experiments

On the basis of results obtained from the preliminary cytotoxicity tests, the two IBU concentrations used during the kinetic experiments in the three cell culture systems were set as follows: the TC_{10} in

296 the corresponding hepatic system, to reveal underlying mechanisms of toxicity, without causing a
297 significant decrease in the number of cells; 1/10 TC_{10}, which should give an insight into
298 pharmacological actions of the drug on cellular level. Eventually, the applied IBU treatment
299 concentrations were 10 µM and 100 µM in PRH and HepaRG cells, and 100 µM and 1000 µM in PHH.
300 Prior to each experiment IBU stock solutions (500- or 1000-fold) were prepared in DMSO and stored
301 at -20°C until needed. Treatment solutions were prepared by diluting the IBU stocks (50 mM and 5
302 mM for PRH and 500 mM and 50 mM for PHH) 1:500 in serum-free culture medium, keeping DMSO
303 concentration at 0.2%. The stock solutions (100 mM and 10 mM) for the HepaRG cells were diluted
304 (1:1000) in medium containing 1% DMSO and 2% FBS to obtain ready-to-use solutions. Aliquots of
305 the daily treatment solution (considered as time 0 samples) and of the stock solutions were stored at -
306 80°C until HPLC analysis.
307 Cell treatment with IBU TC_{10} and 1/10 TC_{10} was initiated by adding 1.5 mL (PRH) or 2 mL (PHH and
308 HepaRG cells) per well of the corresponding ready-to-use solutions or the vehicle (control wells). Per
309 definition, the day of the first exposure was referred to as day 0 and media were changed on a daily
310 basis for 14 days.

311 ***2.8 Sample preparation for HPLC analysis***

312 A sample of cell culture media at time 0 min (t_0) to verify the actual IBU concentration added to the
313 well, and samples of supernatant and cell lysate were collected on day 0/1 and day 13/14 at 5 different
314 time points (TP): 2 min, 30 min, 1 h, 3 h and 24 h for PRH and PHH. In parallel, samples to measure
315 plastic adsorption were prepared on empty wells, as described above. The TP selection was based on
316 intrinsic characteristics of IBU (e.g. 90-99% protein binding, rapid metabolism with half-life 2-4 hours)
317 and the cell systems (e.g. metabolic competence). The *a priori* selected TP were confirmed in a
318 preliminary experiment for PRH and PHH, whereas for HepaRG cells uptake into the cells was
319 delayed. Therefore, to get a better description of the kinetic behaviour in HepaRG cells, the initially
320 selected TP were changed to 30 min, 1 h, 3 h, 6 h and 24 h .

At the selected TP, the supernatant was pipetted into LoBind tubes, and cells were washed twice with PBS. The SW cultured PRH and PHH were scraped and transferred into a new LoBind tube; the well was rinsed with 250 µL methanol, which was added to the same tube. Finally, the cell lysate was homogenised using an in-probe sonicator and the volume was adjusted with methanol to 1 mL. HepaRG cells were scraped in 200 µL PBS and transferred into a new LoBind tube containing 600 µL Methanol. A volume of 200 µL PBS was used to wash the well and after adding it to the same tube, the cell lysate was homogenised (in-probe sonication). All fractions were stored at -80°C until HPLC analysis.

2.9 HPLC analysis of IBU

Methanol was selected as the extraction solvent after checking extraction efficiency: IBU recovery from the 3 media and HepaRG cell lysate was > 95%; for PRH and PHH cell lysate for which the recovery was ~75% an appropriate correction factor was applied for the calculation of IBU content.

Three times volumes methanol were added to the supernatant collected at t_0 and at different TP; the mixture was vortexed for 30 s. All samples, including cell lysates, were centrifuged (Eppendorf 5417R; 5 min, 4°C, 2500 rpm); the upper phase was transferred into amber glass vials for HPLC analysis.

The HPLC system included a PerkinElmer Series 200 analytical pump, a Restek™ Pinnacle ODS Amine C18 column, a PerkinElmer LC 235 Diode Array Detector and the PerkinElmer Totalchrom™ 3.1.2 software for data acquisition and evaluation. IBU identification and quantification was based on the isocratic method described by Hassan et al. (2008) with minor modifications, yielding a better resolution capacity. The method was specific for IBU and not able to detect its major oxidative metabolites (OH- and COOH-IBU) and the glucuronides (data not shown). The mobile phase consisted of a 60:40 (v/v) mixture of methanol and NaH2PO4 (50 mM, pH 6.5), at a flow rate of 0.6 mL/min. The injection volume was 20 µL. The absorption of the eluate was measured continuously at 220 nm (retention time 17 min); the amount of IBU was quantified referring to a calibration straight line (8 concentrations in triplicate, range 1-250 µM; correlation coefficient R2 = 0.978; LOD = 6.2 nM, LOQ = 20.6 nM, CV = 4.5%).

In order to have comparable results across the three biological replicates, the raw data obtained in this study were normalised against the cell number per well. However, cells/well did not vary significantly among the different wells, as determined by measuring protein content (in PHH), or detaching HepaRG cells and counting the number per well (in separate wells close to the ones used for the kinetic analysis), or by high content imaging in separate plates (in PRH) (data not shown); hence, the IBU content in different compartments was expressed as nmol per well.

2.10 Pharmacokinetic Modelling

To describe the *in vitro* pharmacokinetics of IBU, we adapted a three-compartment dynamic model as previously developed for cyclosporine A (Wilmes et al., 2013). The model describes the change in time of the total quantity of IBU in the assay medium (Q_{med}), in cells lysate (Q_{cell}) and bound on proteins such as collagen, if present, (Q_{prot}) in the assay system (Figure 1). The model is generic for simple static system and therefore can be applied to different *in vitro* models as it was shown within the Predict-IV project with appropriate adjustments. The approach is not dissimilar to the one recently published by Armitage et al. (2014), which is a steady-state approximation of an *in vitro* system easy to compute and suitable for high throughput analyses. Since our goal was to perform a kinetic analysis, we developed a dynamic model, including more parameters and their calibration, generating a higher degree of precision in estimates.

No adsorption to plastic vial walls was included, as the obtained experimental data with the three models showed that none occurred for IBU or that it was negligible. Due to sequestration by collagen, for PRH the ”multiple binding” processes to medium proteins, collagen walls were modelled as a single process. For PHH and HepaRG cells there was no evidence of non-specific binding from the blank experiments, thus it was neglected..

Since the experimental data showed that IBU can be metabolised within cells, biotransformation was accounted for in the model. The following three differential equations correspond to the general form of that model:

372 $$\frac{dQ_{med}}{dt} = N_{cell}\left(F_{out}C_{cell} - F_{in}C_{med}\right) + k_2 Q_{prot} - k_1 Q_{med} \quad (1)$$

373 $$C_{med} = \frac{Q_{med}}{V_{med}} \quad (2)$$

374 $$C_{cell} = \frac{Q_{cell}}{N_{cell} V_{cell}} \quad (3)$$

375 where N_{cell} is the number of cells in the assay system, C_{cell} and C_{med} the concentration of IBU in the
376 cells and medium respectively, V_{cell} and V_{med} the volumes of a cells and of the medium respectively, F_{in}
377 and F_{out} the entry and exit rate flows for one cell respectively, k_1 and k_2 the rate constants for binding
378 and unbinding to medium proteins, respectively.

379 $$\frac{dQ_{prot}}{dt} = k_1 Q_{med} - k_2 Q_{prot} \quad (4)$$

380 $$\frac{dQ_{cell}}{dt} = N_{cell}\left(F_{in}C_{med} - F_{out}C_{cell} - \frac{V_{max} \cdot Q_{cell}}{K_m + Q_{cell}}\right) \quad (5)$$

381 where V_{max} is maximal rate of metabolism and K_m the metabolism Michaelis-Menten constant. In Eq. 5
382 a cell volume proportionality difference was used in the definition of K_m rather than concentrations,
383 with no change in the results. The cell volume used for the conversion was 6700 μm^3 for
384 PRH(experimentally determined in-house) and 3400 μm^3 for PHH and HepaRG cells (2000). Details of
385 the parameters used for the modelling are given in Tables S1 and S2 (Supplementary material).
386 For each model the best fit for exchange rates were used: For PRH, the best fit was obtained using
387 first order rates for collagen binding and unbinding, cell entry and exit rate and metabolism (the range
388 of concentration assayed did not include doses high enough to identify a maximum rate of
389 metabolism). For PRH model Eq. 5 above was therefore replaced by its first order equivalent:
390

391 $$\frac{dQ_{cell}}{dt} = N_{cell}\left(F_{in}C_{med} - F_{out}C_{cell} - \frac{V_{max}}{K_m} Q_{cell}\right) \quad (5b)$$

For PHH, no binding to GelTrex™ was observed and its rate was set to zero, first order cell entry and exit rate and saturable metabolism gave the best fit.

For HepaRG cells, the model considers that a large number of biliary cells develop are present in the cell culture. These cells do not metabolise IBU which on the other hand we assumed as able to enter or exit them, at about the same rate per cell as in hepatocytes. We therefore added an equation to describe the potential accumulation of IBU in biliary cells (express as Q_{bcell}). In addition, metabolism is known to increase with time in HepaRG cells (Anthérieu et al., 2010) so we described it as a time-varying first order process. The HepaRG PK model was therefore:

$$\frac{dQ_{med}}{dt} = N_{cell}\left(F_{out}C_{cell} - F_{in}C_{med}\right) + N_{bcell}\left(F_{out}C_{bcell} - F_{in}C_{med}\right) + k_2 Q_{prot} - k_1 Q_{med}$$

where N_{bcell} is the number of biliary cells in the assay system and C_{bcell} the concentration of IBU in those cells.

To fit the model parameters, statistical distributions of their values were obtained by Bayesian numerical calibration (Bois, 2009) with the experimental data provided by the experiments described above. The doses actually measured were used as input to the model, rather than the nominal doses. As usually done with concentration measurements, the data were assumed to be log-normally distributed with geometric means given by the corresponding model predictions and geometric variances sampled from half-normal distributions. Markov-chain Monte Carlo (MCMC) simulations were performed with GNU MCSim version 5.5.0 (http://www.gnu.org/software/mcsim). Two MCMC chains were run in parallel for 50,000 iterations. Their convergence was checked on the last 25,000 iterations using the criterion of Gelman and Rubin (1992). In the case of PHH, there were clear differences between donors, so a hierarchical populations model was used to disentangle variability from uncertainty in parameter estimation (Gelman et al., 1996; Bauer and Guzy, 2007; Bois et al., 2010). Briefly, each 14-day cell culture was fitted individually, yielding one set of parameters for each. However, to stabilize inference and pool information between individual cultures, individuals'

parameter values were supposed to vary following predefined non-uniform distributions. We used log normal distributions. For example, in the case of PHH the cell entry flow rate was supposed to vary log normally around a "population" mean (unknown, to be estimated together with the individual values) with an estimated "population" geometric variance. In the end, a set of parameters was obtained for each individual culture, one set of "population" averages and one set of "population" variances. The (geometric) population means themselves were specified with the prior distributions given in Table 5. The (geometric) population variances were assigned half-normal prior distributions.

Numerical integration of the models was performed with GNU MCSim version 5.5.0. Plots were produced with R, version 2.14.0.

3 Results

3.1 Abiotic processes

IBU was soluble even at the highest used concentration (i.e. stock solutions), both in the vehicle and in the culture media, with recovery of known amount of IBU after solubilisation in the range of 97-116%. IBU was chemically stable under the experimental conditions used (i.e. incubation time, temperature, DMSO and different culture media), with the recovery of known amount of IBU in the range of 92-115%***,*** in glass labware and 94-110% in plastic tubes. In line with this and IBU's low lipophilicity at pH 7.4 (log K_{OW} = 0.8), IBU did not adsorb to the plastic devices used during testing as shown by no IBU extracted by methanol in the *ad hoc* assays, and mass balance close to 100% at the first TP during kinetic experiments (see Figures 2-4, E and F). The media used in the three models contain a sodium-bicarbonate buffer system, which in a 5% CO_2 environment, and maintain an almost constant pH in the physiological range, not altering IBU ionized state during the 14 days of treatment and hence also its lipophylicity (log K_{OW} = 2.48-3.30 at pH 5).

No evaporation leading to cross-contamination among wells occurred, in accordance with IBU low volatility (vapour pressure: 7.08 x 10^{-3} Pa at 25°C).

443 The potentially significant IBU protein binding in the culture media (~99%; Rainsford 2009) was limited
444 by avoiding the use of serum with PRH (replaced with 0.44 mg/mL BSA) and PHH experiments and
445 using 2% FBS (corresponding to 0.8-0.9 mg/mL albumin), in HepaRG cell experiments. This condition
446 did not alter HepaRG cell viability and performance. and was demonstrated not to affect the uptake of
447 amiodarone, a drug with protein binding capacity similar to IBU (Pomponio et al, 2014). IBU recovery
448 (110 ± 10%) from the medium was not influenced by BSA or FBS.
449 In blank experiments, results showed that collagen I (used with PRH) sequestered IBU in a time- and
450 concentration-dependent manner (Table 3). IBU levels in collagen increased on d0 up to 15% after 24
451 h. On d13, the percentages levelled off at around 30% at each time point and concentration,
452 suggesting a saturation of the extracellular matrix. A fraction of the added concentration (content of t_0
453 media) of 15 and 30%, on d0 and d13, respectively, were factored into the intracellular IBU
454 concentrations. Amounts <5% of the nominal concentrations were within the experimental variance
455 and hence were not taken into consideration, also in view of the absence of cells, which are thought to
456 compete with collagen.
457 Analyses of Geltrex™ preparations (used with PHH) showed that negligible binding of IBU to the
458 matrix molecules was found even after repeated exposure with 1000 µM. In accordance with these
459 findings, IBU detected in the corresponding supernatants was in the range 95-108%.
460
461 ***3.2 Kinetic profile of IBU in PRH***
462 The analyses of cell culture media at t_0 revealed that a mean of 8.7 ± 0.7 µM and 85.3 ± 10.3 µM were
463 applied *vs*. a nominal concentrations of 10 µM and 100 µM, respectively, with a CV of 8-12% (N = 20
464 per concentration).
465 At 10 µM (low concentration) a similar IBU kinetic profile was observed on d0 and d13 both for the cell
466 lysate and the supernatant compartments (Figure 2, A and B), indicating that the repeated treatment
467 did not result in any saturation, inhibition or induction phenomena. A rapid and progressive
468 intracellular uptake of IBU was evident from 2 min to 1 h. After a steady state from 1 to 3 h the amount

of IBU in the cell lysates decreased up to 24 h (Figure 2, A). In parallel, a non-proportional decrease was observed over time in the supernatant, indicating a steady state from 1 to 3 h followed by a drop to 3 and 12% of applied IBU at 24 h of d0 and d13, respectively (Figure 2, B). The non-quantitative correspondence between the decrease in the supernatants and the increase in the cell lysates over time was confirmed by the calculation of the mass balance (Figure 2, C), that is the total amount of IBU recovered in all the different compartments at a given time point, compared to the initially added amount. It indicated a ”apparent” loss of IBU over time (striped bars in Figure 2, C), which could be attributed to biotransformation processes (slightly lower on d13).

At 100 µM IBU (high concentration), the kinetic profile was similar (Figure 2, D and E): the low to high concentration ratio (1/10) was maintained in the IBU amount measured in cell lysates, increasing on both days from 2 min to 1 h. A steady state (1 to 3 h) followed and then a decrease to a minimum of ~6 and 13% at 24 h (d0 and d13, respectively) (Figure 2, D).

Figure 2 E illustrates the continuous decrease of IBU in the supernatant showing a steady state from 1 to 3 h and a drop at 24 h to ~18 (day 0) and 36% (day 13) of the initial amount. The relative distribution in the different compartments again showed an apparent ‘loss’ in mass balance (Figure 2, F).

3.3 Kinetic profile of IBU in PHH

When the kinetic profile of IBU was followed in PHH, the different human donors were firstly evaluated individually rather than as biological replicates, due to high intra-individual variability. Although the absolute values were different, representing the expected variability among individuals, analysed donors exhibited a comparable trend: on this basis, distinct donors were handled as biological replicates.

This high variability (translated in high SD bars) was very likely attributed to differences in metabolism, although the hepatic pathology of the donors might also have contributed. In order to characterise the competence of PHH to metabolise IBU, some CYP specific activities were measured in PHH from

each donor (Table 4). Of particular interest was CYP2C9, which is the main CYP involved in IBU metabolism. PHH from Donor B1032 were expected to show a low level of oxidative IBU metabolism. Indeed, after 3 days of repeated exposure, a high degree of cytotoxicity was observed at both concentrations. As a result, the experiment with PHH from Donor B1032 was stopped because it did not meet the criteria set in the experimental design, i.e. high concentration corresponding to TC_{10}, thus assuming a nearly constant number of cells throughout the experimental treatment. As a consequence, biokinetic data reported on d13 included only samples from PHH from the Donors S1045 and B1050.

The mean IBU concentration at T_0 was 97.8 ± 10.0 µM and 803.7 ± 78.6 µM vs the nominal 100 µM and 1000 µM, respectively, with a CV of 2-20%% with N = 20 (for each concentration).

At 100 µM (low concentration) no differences in IBU kinetic profile were evident between d0 and d13. IBU uptake by PHH was very rapid, reaching maximum values (5% of the applied concentration) after 2 min at d0 and after 30 min at d13 (Figure 3, A). Subsequently, a steady state up to 3 h was followed by a decrease to a minimum of ~ 1% at 24 h. IBU content in the PHH supernatant samples showed an almost continuous decrease over time with a less pronounced steady-state at early times. In no case the intracellular amount and the decrease in the supernatant were quantitatively correlated (Figure 3, B). The sum of the amount of IBU found in the cell lysates plus supernatants declined quite rapidly starting from the first time point on d0 (Figure 3, C); on d13 the decreasing rate was slower, but after 24 h in both cases very low IBU values were attained (10.2 ± 4.8% on d0 and 8.6 ± 4.3% on d13). As evidenced by the relative IBU distribution in the different compartments (Figure 3, C), IBU content in the cell lysate never exceeded 5%, the amount measured at the steady state, supporting the occurrence of a very efficient metabolism of the parent compound by PHH.

The kinetic profile in the cell lysate over 24 h for the PHH treated with 1000 µM (high concentration; Figure 3, D) showed a different pattern; the high/low intracellular concentration ratio was > 10 fold. After a very rapid uptake into the cells, at 2 min the amount of IBU remained at a constant level (around 1-3%) at d0; although very variable among donors, the trend was similar at d13. In contrast,

521 the supernatants revealed a slight decrease at earlier time points, (Figure 3, E), then IBU levels were
522 almost constant up to 24 h. On d0 the relative distributions showed a decline in total recovered IBU
523 from cells plus supernatant (Figure 3, F), but never below 50%. After repeated exposures, the 3 h time
524 point corresponded to very low recovery and nearly no variability between samples and donors,
525 contradicting results obtained both at previous time points as well as at 24 h, when results were
526 consistent with the overall kinetic behaviour were obtained. The data at 3 h were interpreted as a
527 technical problem, since at 24 h cell were perfectly viable and CYP2C9 activity was present (data not
528 shown), therefore the low metabolic capacity at 24 h cannot be attributed to cytotoxicity. Under these
529 conditions, the decrease in IBU mass balance was around 20-30% with most IBU recovered in the
530 supernatant at 24 h, as shown by its relative distribution (squared bars in Figure 3, F). This suggested
531 a saturation of IBU biotransformation at 1000 µM, which could be expected at such a high
532 concentration.
533
534 ***3.4 Kinetic profile of IBU in HepaRG cells***
535 The actual IBU mean concentration in the media at t_0 was 7.8 ± 1.2 µM and 85.4 ± 4.2 µM versus the
536 nominal 10 µM and 100 µM, with a CV between 5 and 16% with N = 20 (for each concentration).
537 In HepaRG cells treated with 10 µM IBU, the uptake started at 30 min, reaching a plateau after 3-6 h
538 (Figure 4, A). In the supernatant a continuous decrease in IBU content was measured both on d0 and
539 d13, reaching a similar amount at 24 h (Figure 4, B). The relative distribution showed a continuous
540 and constant decrease of IBU mass balance in the course of 24 h both on d0 and d13. Although the
541 reduction rate was higher after repeated exposure (Figure 4, C), likely attributable to higher level of
542 CYP2C9 on d13.
543 After treatment of HepaRG cells with 100 µM IBU (high concentration), the measured kinetic profile
544 was similar: in the cell compartment on d0 and d13 IBU content remained low, under 0.5 nmol/well
545 (Figure 4, D). A gradual decrease in IBU content was observed in the supernatants on d0 higher on
546 d13, but never quantitatively related to the increase in intracellular content (Figure 4, E). The total IBU

recovery showed a continuous decrease over time on both days (Figure 4, F), although significantly lower on d13. This suggests a possible increase in the CYP2C9-related biotransformation capacity of this cell model over time. The IBU amount in the supernatants was 10 fold higher than that measured at 10 µM IBU, respecting the high/low concentration ratio, whereas it was < 10 in cell lysates.

3.5 Pharmacokinetic Modelling

Figure 5 shows the measured and modelled amount of IBU in the medium and cell lysates of PRH cultures over the repeated treatment of 14 days. Since IBU was applied daily, the concentration was reset to nominal medium concentrations every 24 h. It was assumed that IBU content in cell lysate corresponds to intracellular concentrations; the assumption was nevertheless reasonable, since, based on its low lipophilicity (log K_{OW} = 0.8 at pH 7.4), IBU is unlikely sequestered within membranes. The data were well matched by the model and fairly consistent across replicates. The various curves were very similar, showing that there was reasonable uncertainty in the model predictions (the estimate of residual uncertainty corresponded to a CV of 30%).

A dynamic equilibrium was reached already after the first day. The ratios Q_{cell} over Q_{med} were close to 0.2, but the total cellular volume was about a 1000th of the medium volume, so the ratio of concentrations was about 200. As a result, cells had a 200 times higher IBU concentration than the medium.

A similar model fitted for PHH data is shown in Figure 6. The PHH from Donor B1032 proved to be very sensitive to IBU toxicity at the applied concentration, thus only data from the first day were obtained. Here, significant inter-individual variability was noted, thus a population approach was used to analyse the data deriving from PHH. The fits were quite good, but uncertainty (reflected by the spreading of the simulated curves) was as expected higher compared to PRH, resulting from underlying measurement errors and modelling approximations (the estimate of residual CV being 40%). In two PHH donors, the intracellular quantity of IBU was noticeably higher than predicted. In the data sets of PHH from Donors S1045 and B1050, a dynamic equilibrium was reached practically on

d1, as previously seen in the modelling of the PRH data. The IBU amounts in PHH of the 100 µM group were underestimated by the model. This could be due to systematic error in IBU recovery, non-linearity in cellular uptake of efflux, lower metabolism at IBU low concentration, or some unknown mechanism. More data would be required to identify the actual cause of this observation.

The data and model fit for HepaRG cells are given in Figure 7. The applied model layout was similar to that previously described for PRH and PHH. As for the PHH data, the three replicates were analysed in a population framework, but reflecting inter-experiment variability instead of inter-individual differences between donors. There was a rather large proportion of non-detectable levels in the low-dose experiments with substantial uncertainty (residual uncertainty: CV = 45%). Furthermore, an underestimation of some data points at 10 µM concentration in cell lysates was observed as previously observed for the PHH. These results seem however, globally consistent with those of the other two culture systems. For both PHH and HepaRG cells, the ratios Q_{cell} over Q_{med} were close to 0.01, indicating that those cells had only a 10 times higher IBU concentration compared to the medium – that is much less than in PRH, likely due to more efficient metabolism.

The posterior parameter distributions obtained after MCMC sampling for the "average" parameters are summarised in Table 5. They characterise the kinetic behaviour observed an average experiment for PRH or HepaRG cells, or of the cells of an average donor in PHH. Given the dose assayed in the various systems and the cell capabilities, we could estimate a maximum rate of metabolism only in PHH. The low-dose metabolic clearances, V_{max} / K_m, could be estimated in all systems with very good precision in PRH (5% CV) and less so in PHH and HepaRG (50% CV). The PRH have clearly lower clearance rates, which explained the much higher IBU concentrations observed in those cells. Clearance rates were similar for the different human cells, given the significant variability between donors and experiments. In HepaRG cells, metabolic clearance is known to increase with time, and we estimated (slope parameter α) that it was about 60% higher after 14 days compared to the start (Table 5).

4 Discussion

To overcome the imbalance between rising costs and declining approvals of new drugs, the pharmaceutical industry is under pressure to improve the effectiveness of the drug development process, and reduce the current high attrition rates. Toxicity testing is crucial, but represents a time- and resource-consuming step; in addition human ADRs to the liver are often difficult to predict with existing animal models. These considerations, besides the ethical considerations related to the use of laboratory animals, promote the development of more suitable *in vitro* methods. This paper addresses the importance of measuring *in vitro* biokinetics, generally not taken into account even in well-established experimental *in vitro* models, in order to improve the predictability of *in vitro* data, and to be used as input in PBPK models (Coecke et al., 2013). We used three different hepatic cellular systems, with IBU as a model compound.

IBU is a widely used as analgesic/antipyretic agent, available by prescription and over-the-counter; at therapeutic doses it is reported, although rarely, to induce ADRs to the liver (Bennett et al., 2009; Rodríguez-González et al., 2002; Laurent et al., 2000). Among them, sub-acute hepatic failure, hepatitis C cases and significant increase in transaminases (O'Connor et al., 2003). In humans, the first step in IBU metabolism is catalysed by CYP2C9 and to a lower extent by 2C8 (Chang et al., 2008), to form 2-Hydroxy-IBU and Carboxy-IBU, as major detoxification metabolites (Hamman et al., 1997). The rat CYP2c6 and 2c11, orthologs of human CYP2C9 (Vecera et al., 2011), are thought to be involved in these oxidative reactions (Hu et al., 2011; Kapil et al., 2004). IBU and its oxidative non-toxic metabolites are conjugated with glucuronic acid by different UGTs *in vitro* in rat or human hepatocytes (Dong and Smith, 2009) and *in vivo* (Spraul et al., 1992) and then excreted, mainly in urine.

As the first step we checked factors possibly affecting the bioavailability of IBU under the experimental conditions used, and showed that IBU was readily soluble and chemically stable, neither cross-contamination among wells nor adsorption to plastic devices occurred. The use of FBS or albumin in the medium was reduced to a minimum, i.e. not affecting IBU recovery or total bioavailability.

Furthermore, no IBU was physically sequestered into the Geltrex™ used in PHH SW culture. By contrast, we found a time- and concentration-dependent amount of IBU in collagen I used in PRH cultures. This finding was relevant because physically sequestered IBU could be assumed as intracellular content, and erroneously interpreted as bioaccumulation.

The study design was similar to that of the *in vivo* kinetic testing (OECD Test Guideline 417). The comparison of kinetic behaviour obtained between single and repeated treatments allowed the study of inhibition, saturation or induction of cellular influx/efflux, metabolism and bioaccumulation processes.

The used 100 µM IBU corresponds to 20.6 µg/mL, which approximates the recently reported human peak plasma concentration (C_{max} = 22.6 ± 5.6 µg/mL) measured upon oral intake of 400 mg IBU (Vilenchik et al., 2012) in the low range of therapeutic doses. Hence, the cross-model comparison of the three hepatic systems was conducted at 100 µM IBU.

The obtained modelled results showed a coherent picture for IBU *in vitro* biokinetics across the three models: (i) IBU quickly entered cells; (ii) after multiple exposures a dynamic equilibrium was reached within one or two days; (iii) the decrease in the medium was not quantitatively related to the increase in the cell lysate fractions. All three models showed a significant capacity in metabolising IBU; therefore, cells removed (by biotransformation) the parent compound in 24 h, resulting in no bioaccumulation after daily treatment for 14 days This is in line with information from *in vivo* pharmacokinetic data in humans showing that 60% of a given dose was excreted within the first 24 h (Adams et al., 1969). Although the experimental data were available only on d0 and d13, modelling allowed the prediction of the daily kinetic behaviour along the treatment period.

The uptake of IBU was likely due to passive transport through the cell membrane, being very rapid and not saturable: and assumed to be a "first order" kinetic process. Our assumption was supported by experiments conducted on Caco2 cells indicating a passive transport (data not shown) and by data showing that in rats IBU is an Oatp1a1 inhibitor (Zhang et al., 2013) and in human cells IBU inhibits OATP1B1 or OATP1B3, without being a substrate (Kindal et al., 2011). The modelling showed that

651 cellular uptake was much faster compared to its output; since, once entered, the drug was rapidly
652 metabolised, the efflux back in the medium compartment, if any, was considered negligible. An initial
653 slower intracellular uptake characterised HepaRG cells (paralleled by a slower decrease of IBU
654 content in the medium fraction, Figure 8 B at d0). It could be attributed to the presence of 2% FBS in
655 the medium binding IBU. However, considering protein binding as a fast process, despite the cell
656 uptake rate was initially slower, the total amount of IBU entering the cells within 24h was the same
657 (>90% IBU ”lost” in the mass balance estimate). The consequence could be a slight overestimation in
658 the HepaRG clearance.
659 After single exposure, the steady state was reached rapidly in PHH (2-30min) > PRH (1-3h) >
660 HepaRG (3-6h). The higher intracellular concentration detected in PRH than in human derived cells
661 (Figure 8 A, C) at the steady state could be due to the different metabolic clearance rates, which in the
662 rat model are one order of magnitude lower compared to the human-derived cells (Table 5). The
663 difference in metabolic clearance was shown also *in vivo* (Adams et al., 1969), with a more efficient
664 IBU metabolism in humans compared to the rat.
665 The HepaRG cells showed lower IBU concentrations in the cell lysates than PHH, particularly after
666 repeated treatment. This could be due, at least partially, to the 60% increase over time of IBU
667 metabolic clearance in HepaRG cells (Table 5) which was not a compound-specific effect. Indeed,
668 higher levels of CYP2C9 activity at 14 days of culture have been reported (Anthérieu et al., 2010) as
669 well as for other CYPs (Jossé et al., 2008). In addition, transcriptomics measurements indicated no
670 significant increase in CYP2C9 mRNA indicative of induction by IBU treatment, although few genes
671 involved in drug metabolism were increased in control cells on d13 (data not shown). The absence of
672 induction throughout the course of repeated exposure in the three models was in accordance with *in*
673 *vivo* data (Mills et al., 1973).
674 The efficiency of IBU metabolism seemed to be a strong determinant for its toxicity in hepatocytes.
675 Here, PRH, with a slower metabolic clearance, showed a higher toxicity compared to PHH although,
676 due to the high inter-individual variability in PHH, the obtained TC_{10} cannot be considered as

representative of the average human population. Indeed, PHH from Donor B1032, characterised by low CYP2C9 activity and thus, by a low IBU detoxification, experienced a high cytotoxicity, leading to experiment termination after 3 days. It is unclear why TC_{10} in HepaRG was lower than PHH and similar to PRH, although having a much higher metabolic clearance. A possible explanation, beside the lower number of cells (50% of the population is made of biliary cells) is a different balance between phase-1 and phase-2 metabolism in the three models. Although not measured and modelled in this work, glucuronidation has been also shown to play a role in the metabolic disposition of most 'profens' Since species differences have been described in glucuronidation rates in liver microsomes (rate ranking: man, monkey, dog, rabbit and rat) (Magdalou et al., 1990), phase 2 metabolism may contribute to differences in the total biotransformation as well as to IBU-induced toxicity among species and between the human-derived models. Indeed, 'profen'-acyl glucuronides have been shown to form covalent protein adducts in rat hepatocytes (Dong and Smith, 2009), contributing to hepatic toxicity. Although IBU is glucuronidated less efficiently than other compounds of the same family (Magdalou et al., 1990) and IBU-acyl glucuronides are considered less reactive among those formed with other 'profens' (Castillo et al., 1995), their higher formation in the PHH from Donor B1032, due to the scant CYP2C9 activity, may be responsible for the pronounced toxicity.

Here, the disappearance of the parent compound over time was used as a measure for metabolic clearance. The detection of the different metabolites, including glucuronides, would have delivered valuable additional information whether DMEs are differentially active in the three *in vitro* systems, accounting for the observed differences. The use of DME-phenotyped PHH could further help in the full understanding of metabolism-related IBU toxicity. Overall, the observed metabolism-dependent toxicity could be a hint to the rather idiosyncratic nature of IBU-induced liver injury in patients (Bennett et al., 2009; Rodríguez-González et al., 2002; Laurent et al., 2000).

The importance of the intracellular concentration for concentration-response extrapolations was comprehensively described by Groothuis et al. (2013). Here we provide evidence that the use of nominal concentrations would have resulted in an overestimation of the actual treatment (up to 20%).

More importantly, the content in the cell lysates over time appeared to be different in the three hepatic models tested. The disappearance of the test item in the culture medium which was, from a technical point of view, less demanding than testing the cell lysate fraction, showed no major differences in the three hepatocyte models after repeated treatment. Hence, monitoring the biokinetic profile in this single compartment did not allow the drawing of any clear conclusions. Consequently, the measurement of the compound in the cell lysates and the calculation of the relative distributions using mass balance values, were crucial parameters to be monitored.

Overall, in terms of species-differences, our data reflected the *in vivo* situation. Here, consideration of dose-normalised pharmacokinetic parameters such as C_{max} (rat < human), T_{max} (rat < human) and $t_{1/2}$ (rat ~ human) revealed that at 100 µM IBU was metabolised as *in vivo* more efficiently in humans compared to rats (Hu et al.,2011; Kapil et al., 2004; Teng et al., 2003; Adeyeye et al., 1996). Although PRH appeared to give reproducible results, as expected by the species-specificity, IBU intracellular concentration in PRH did not correspond to IBU intracellular concentrations found in human cells under similar exposure conditions, suggesting a poor predictivity from this species. A high variability was shown by PHH from the different donors, although modelling made it possible to account for (and in reduce) variability between donors. The residual uncertainty (Table 5 and in model prediction Figures) was overall about 10%. The number of donors tested was limited, but the use of an appropriately higher number of donors could provide an estimate of human variability, which may be considered an advantage, representing the inter-individual differences observed clinically. HepaRG cells gave very reproducible data and although a large population (~50%) of non-metabolising biliary cells is present, they did not bioaccumulate IBU. This was in line with previous data showing that cryopreserved HepaRG cells are a valuable tool for kinetic prediction of CYP substrates (Zanelli et al., 2012).

5 Conclusion

The consideration of the biokinetic profiles could help explaining specific observations, i.e. transcriptome, proteome and enzyme induction/inhibition, and support a more holistic biological interpretation. Biokinetics is the link between the applied dose and the observed effects. Modelling these effects taking into account the time course of concentrations is at the root of time-based simulations of effects, i.e. pharmacokinetic/pharmacodynamic (PKPD) modelling, allowing one to make predictions from *in vitro* to *in vivo* effects. Finally, the transformation of an *in vitro* NOAEC to a relevant *in vivo* NOAEL would greatly benefit drug discovery and reduce the chance of hepatotoxic compounds making it through to clinical trials or on to the market.

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Figure Legends

Figure 1. Schematic representation of the three-compartment model used for ibuprofen *in vitro* pharmacokinetics.

Figure 2. Kinetic profile of ibuprofen (IBU) (nmol/well) in primary rat hepatocytes (PRH) (A, D) and culture supernatants (B, E) after single (day 0 (d0) – dashed line) and repeated (day 13 (d13) – solid line) treatment with 10 µM IBU (A, B) or 100 µM IBU (D, E). Relative distribution (%) of IBU in the different analysed PRH fractions at the indicated time points for d0 and d13, supernatant (squared) and cell lysate (blank) as well as the apparent loss (striped) for the low concentration (10 µM, C) and high concentration (100 µM, F). Values are given as mean of three biological replicates +/- SD (standard deviation); each sample was run in two technical replicates. The significance of the results was analysed by the Student t test ($p<0.05$): the letter a indicates statistically significant differences between each data point and the first time point; the letter b indicates statistically significant differences between each data point and the immediately precedent time point.

Figure 3. Kinetic profile of ibuprofen (IBU) (nmol/well) in primary human hepatocytes (PHH) (A, D) and culture supernatants (B, E) after single (day 0 (d0) – dashed line) and repeated (day 13 (d13) – solid line) treatment with 100 µM IBU (A, B) or 1000 µM IBU (D, E). Relative distribution (%) of IBU in the different analysed PHH fractions at the indicated time points for d0 and d13, supernatant (squared) and cell lysate (blank) as well as the apparent loss (striped) for the low concentration (100 µM, C) and high concentration (1000 µM, F). As the PHH from Donor B1032 evidenced a high cytotoxicity after 3 days of treatment with IBU, values are given as mean of two (d13) or three (d0) biological replicates +/- SD; each sample was run in two technical replicates.

Figure 4. Kinetic profile of ibuprofen (IBU) (nmol/well) in HepaRG cells (A, D) and culture supernatants (B, E) after single (day 0 (d0) – dashed line) and repeated (day 13 (d13) – solid line) treatment with

10µM IBU (A, B) or 100µM IBU (D, E). Relative distribution (%) of IBU in the different analysed HepaRG cell fractions at the indicated time points for d0 and d13, supernatant (squared) and cell lysate (blank) as well as the apparent loss (striped) for the low concentration (10 µM, C) and high concentration (100 µM, F). Values are given as mean of three biological replicates +/- SD; each sample was run in two technical replicates. The significance of results was analysed by the Student t test ($p<0.05$): the letter a indicates statistically significant differences between each data point and the first time point; the letter b indicates statistically significant differences between each data point and the immediately precedent time point.

Figure 5. Amount of ibuprofen (IBU) in nmol, measured in the medium (triangles) and cell lysates (circles) of primary rat hepatocytes (PRH) cultures, superimposed with the best (maximum posterior) fit of the three-compartment pharmacokinetic model (thick black line) and a set of predictions generated from random set of posterior parameter values (thin black lines). IBU was applied daily at either high (100 µM corresponding to 150-200 nmol per day; top row A) or low concentration (10 µM corresponding to 15-20 nmol per day; bottom row B). Three biological replicates were analysed (in column, 1-3).

Figure 6. Amount of ibuprofen (IBU) in nmol measured in the medium (triangles) and cell lysates (circles) of primary human hepatocytes (PHH) cultures, superimposed with the best (maximum posterior) fit of the three-compartment pharmacokinetic model (thick black line) and a set of predictions generated from random set of posterior parameter values (thin black lines). IBU was applied daily at either high (1000 µM corresponding to 1.5-2 µmol per day; top row A) or low concentration (100 µM corresponding to 150-200 nmol per day; bottom row B). Three donors were analysed (in column, 1-3) in population pharmacokinetic framework. Experiment with PHH from Donor B1032 was stopped, due to the toxicity of IBU to those cells.

Figure 7. Amount of ibuprofen (IBU) in nmol measured in the medium (triangles) and cell lysates (circles) of HepaRG cultures, superimposed with the best (maximum posterior) fit of the three-compartment pharmacokinetic model (thick black line) and a set of predictions generated from random set of posterior parameter values (thin black lines). IBU was applied daily at either high (150-200nmol per day; top row A) or low concentration (10-20nmol per day; bottom row B). Three replicate experiments were analysed (in column, 1-3) in population pharmacokinetic framework.

Figure 8. Cross model comparison between primary rat hepatocytes (PRH) (dashed line), primary human hepatocytes (PHH) (squares, solid line) and HepaRG cells (triangles, solid line): Biokinetic profile of ibuprofen (IBU) (nmol/well) in cell lysates (A, C) and culture supernatants (B, D) after single (day 0 (d0) – upper panel: A, B) and repeated (day 13 (d13) – lower panel: C, D) treatment with 100 µM IBU. The five time point correspond to 2 min, 30 min, 1 h, 3 h and 24 h for PRH and PHH, and 30 min, 3 h, 6 h, 12 h and 24 h for HepaRG cells. Values are given as mean of three biological replicates +/ SD; each sample was run in two technical replicates.

Tables

Table 1. Detailed information on the donors of liver resections utilised within this study.

Donor	Sex	Age	Pathology	Medication
S0302V[a]	Male	61	Sigmoid adenocarcinoma	Lypanthyl, Stagid, Cotareg, Insulin, Sotalex
B1032	Male	42	Echinococcosis alveolaris	none
S1045	Male	75	Hepatic tumor	Atenolol, Kardegic, Ramipril, Inspra, Rasilez
B1050	Male	63	Hydatid Cyst	none

[a] This sample was used only for a preliminary assessment of IBU cytotoxicity. It was not further used for the kinetic experiments,

Table 2. Ibuprofen (IBU) concentrations applied to primary rat hepatocytes (PRH) cultures in different conditions for determination of the concentration that causes 10% cytotoxicity.

Culture condition	**[IBU] in µM**					
96-well (ML – 24h)	500	1000	1500	2000	3500	5000
24-well (SW – 1, 3, 14d)	1	10	100	500	1000	2000
6-well (SW – 14d)	10	500	1000			

Table 3. IBU sequestered by collagen I used for primary rat hepatocytes (PRH) long-term cultures.

		IBU in %	
d0	2 min	1.6 ± 1.3 (101.3 ± 1.3)	3.4 ± 0.4 (98.2 ± 0.3)
	24 h	13.6 ± 2.7 (97.6 ± 5.8)	15.1 ± 0.9 (103.4 ± 2.2)
d13	2 min	29.3 ± 0.6 (94.3 ± 3.1)	28.5 ± 1.3 (99.8 ± 5.6)
	24 h	31.4 ± 3.0 (104.3 ± 0.9)	26.4 ± 1.7 (106.3 ± 1.7)
	Nominal concentration	**IBU 10µM**	**IBU 100µM**

Values are given in per cent of IBU detected in collagen I, compared with IBU detected in cell media at T_0. Figures in parentheses are the total IBU recovery (media + collagen I). Results are expressed as mean ±standard deviation (SD) obtained on three replicates.

d0 = day 0; d13 = day 13

Table 4. Cytochrome P450 activities of primary human hepatocytes (PHH) from the three donors used for the biokinetic studies.

	Activity in pmol/min/mg protein				
Donor	**CYP1A1/2**	**CYP2B6**	**CYP3A4**	**CYP2C9**	**CYP2D6**
B1032	< LOQ	5.33	0.104	3.81	0.281
S1045	0.221	23.1	33.4	29.9	3.36
B1050	0.140	22.1	< LOQ	15.1	0.809

The specific activity of cytochrome P450s (CYP) in the hepatocyte preparations deriving from the three different donors was assessed one day after seeding. Results were expressed as average of two technical replicates.

Table 5. Summary statistics of the posterior "population" mean parameters distribution of the *in vitro* ibuprofen (IBU) kinetic models in the three cellular systems.

Parameter	PRH values[a]	PHH values	HepaRG cell values
F_{in}	230 ± 25 [180, 290]	259 ± 75 [130, 440]	- [d]
F_{out}	24 ± 2.9 [17, 30]	45.8 ± 15 [23, 84]	- [d]
k_1	$3.5\times10^{-4} \pm 7.4\times10^{-5}$ $[2\times10^{-4}, 5.2\times10^{-5}]$	- [d]	- [d]
k_2	$1.4\times10^{-3} \pm 3.3\times10^{-4}$ $[7.4\times10^{-4}, 2.2\times10^{-3}]$	- [d]	- [d]
V_{max}	- [b]	$5.7\times10^{-9} \pm 3\times10^{-9}$ $[2.2\times10^{-9}, 1.4\times10^{-8}]$	- [b]
V_{max} / K_m	0.47 ± 0.02 [0.42, 0.5]	6.5 ± 3.0 [3.0, 15]	13 ± 6.5 [5.4, 30]
α	- [c]	- [c]	$5.1\times10^{-7} \pm 1.4\times10^{-7}$ $[2.5\times10^{-7}, 8\times10^{-7}]$

These posterior distributions were obtained by model calibration with the data. For PRH they characterise to a typical experiment; while for PHH they correspond to the geometric mean of a virtual population of hypothetical donors. For HepaRG cells they represent the geometric mean of a virtual set of experiments.

[a] (mean ± SD) [2.5 and 97.5 percentiles of the posterior distribution].

[b] not applicable (first order kinetics).

[c] not applicable (slope set to zero).

[d] not applicable (fixed value parameter).

Supplementary Data

Table S1. Population mean parameters descriptions, set values or statistical distributions of the *in vitro* IBU kinetic model for PHH. The posterior distributions were obtained by model calibration with the data and represent the geometric mean of a virtual population of hypothetical donors.

Parameter	Description	Units	Value or Prior distribution
N_{cell}	number of hepatocytes	-	2×10^6
V_{med}	volume of assay medium	μm^3	2×10^{12}
V_{cell}	volume of a cell	μm^3	3400 [c]
F_{in}	cell entry rate flow [a]	$\mu m^3.sec^{-1}$	U (0, 500) [d]
F_{out}	cell exit rate flow [a]	$\mu m^3.sec^{-1}$	U (0, 10^6)
k_1	GelTrex™ binding rate constant	sec^{-1}	0
k_2	GelTrex™ unbinding rate constant	sec^{-1}	0
V_{max}	maximum rate of metabolism [a]	$nmol.sec^{-1}$	U (0, 10^{-7})
V_{max} / K_m	low-dose metabolic clearance [a,b]	$\mu m^3.sec^{-1}$	U (0, 10^6)

[a] Per hepatocyte.

[b] At each iteration, V_{max} was divided by this parameter to compute the K_m value to use in Eq. 5.

[c] Lodish et al., 2000.

[d] U (min, max): uniform distribution.

1008 Table S2. Population mean parameters descriptions, set values or statistical distributions of the *in vitro*
1009 IBU kinetic model for HepaRG cells. The posterior distributions were obtained by model calibration
1010 with the data and represent the geometric mean of a virtual set of experiments.

Parameter	Description	Units	Value or Prior distribution
N_{cell}	number of hepatocytes	-	10^6
V_{med}	volume of assay medium	μm^3	2×10^{12}
V_{cell}	volume of a cell	μm^3	3400 [b]
F_{in}	cell entry rate flow [a]	$\mu m^3.sec^{-1}$	150
F_{out}	cell exit rate flow [a]	$\mu m^3.sec^{-1}$	30
k_1	protein binding rate constant	sec^{-1}	0
k_2	protein unbinding rate constant	sec^{-1}	0
$V_{max}(t_0) / K_m$	initial metabolic clearance [a]	$\mu m^3.sec^{-1}$	U (0, 10^6) [c]
α	clearance slope factor	sec^{-1}	U (0, 10^{-6})
N_{bcell}	number of biliary cells	-	10^6

1011 [a] Per cell.
1012 [b] Lodish et al., 2000.
1013 [c] U (min, max): uniform distribution.